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Transmembrane Helix 12 of the *Staphylococcus aureus* Multidrug Transporter QacA Lines the Bivalent Cationic Drug Binding Pocket▽

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An acidic residue in transmembrane segment (TMS) 10 is important for recognition of bivalent cationic substrates by the QacA multidrug transporter. Remarkably, an acidic residue in TMS 12 compensated for the absence of such a residue in TMS 10, suggesting that TMS 12 is a component of the bivalent cation-binding region.

The *Staphylococcus aureus* multidrug transport protein QacA is a member of the drug:H^+ antiporter (DHA) 2 family of the major facilitator superfamily and is composed of 514 amino acid residues organized into 14 α-helical transmembrane segments (TMS) (Fig. 1) (4). The multidrug recognition profile of the QacA transporter is distinct from that of the closely related QacB protein, in that QacA confers high-level resistance to both monovalent and bivalent cationic antimicrobials, whereas QacB provides poor resistance to bivalent cations (9). Previous studies have shown that of the six amino acid differences between QacA and QacB, only one, the incorporation of an aspartic acid residue at position 323 (D323) in TMS 10 of QacA in place of an alanine at this position in QacB, is required to extend the substrate recognition profile of QacA to include bivalent molecules (Fig. 1) (9). In the absence of D323, an acidic residue in one of two other positions in TMS 10, 320 or 322, can also promote bivalent substrate recognition (9, 11). These positions are thought to line the binding site for bivalent cationic drugs in QacA, situating the acidic residues such that they can participate in electrostatic interactions with bivalent substrates (7, 9, 11).

Similar to QacA, electrostatic interactions are thought to be highly important in the binding of cationic drugs by other multidrug-binding proteins. For example, crystal structures of the QacA regulatory protein, QacR, which binds a range of monovalent and bivalent cationic antimicrobial compounds similar to that bound by QacA, show that the positive charges carried by these compounds are typically neutralized by one of five glutamic acid residues which line the drug-binding pocket in QacR (8, 10). Additionally, neutralization of E26 in TMS 1 of the *Escherichia coli* DHA1 family multidrug transporter MdfA disrupts the capacity of MdfA to recognize a large number of cationic substrates (2, 5), and the neutralization of either one of two acidic residues in the lactococcal DHA1 family multidrug transport protein LmrP abolishes the transport of bivalent, but not monovalent, cationic substrates by this protein (6).

A study investigating the genetic diversity and distribution of genes encoding QacA/B transport proteins within clinical *S. aureus* strains from Japan identified three variants which encoded proteins containing no acidic residues in TMS 10 but a glutamic acid residue in place of a glycine at amino acid position 377 (G377E) in TMS 12 (Fig. 1) (3). Due to the unfavorable energetic cost, charged residues are unlikely to be buried within the membrane without conferring a selective advantage on the protein. Therefore, the maintenance of the G377E alteration within three distinct QacA/B variants was intriguing. To investigate whether the properties of the amino acid residue at position 377 could influence the substrate specificity of QacA, the phenotypic effects of acidic and neutral substitutions at this position were determined in the presence of D323 and after its neutralization by cysteine substitution.

Site-directed mutagenesis using the Stratagene QuikChange method was used to incorporate glutamic acid, aspartic acid, glutamine, or cysteine substitutions for G377 in both wild-type QacA and D323C QacA mutant templates (11). Each of the eight mutants was expressed at close to wild-type levels in *E. coli* DH5α cells, as determined by Western analysis with a QacA-specific antiserum (Table 1). The ability of the mutants to confer resistance to six QacA substrates, selected as representatives from different chemical classes and including both monovalent and bivalent compounds, was examined using MIC analysis. As shown previously (11), the QacA D323C mutant conferred only background levels of resistance to each of the bivalent drugs tested while maintaining wild-type or greater levels of resistance to monovalent substrates (Table 1). Although the rhodamine 6G resistance levels conferred by the D323C-G377C/D/E/Q QacA mutants were lower than those conferred by the D323C mutant, these mutant proteins retained a high overall capacity to confer resistance to monovalent cationic substrates (Table 1). Furthermore, neither of the two neutral amino acid substitutions for G377 in the D323C QacA background (viz., D323C-G377C and D323C-G377Q) caused a significant increase in the overall level of bivalent drug resistance observed, although the level of resistance to

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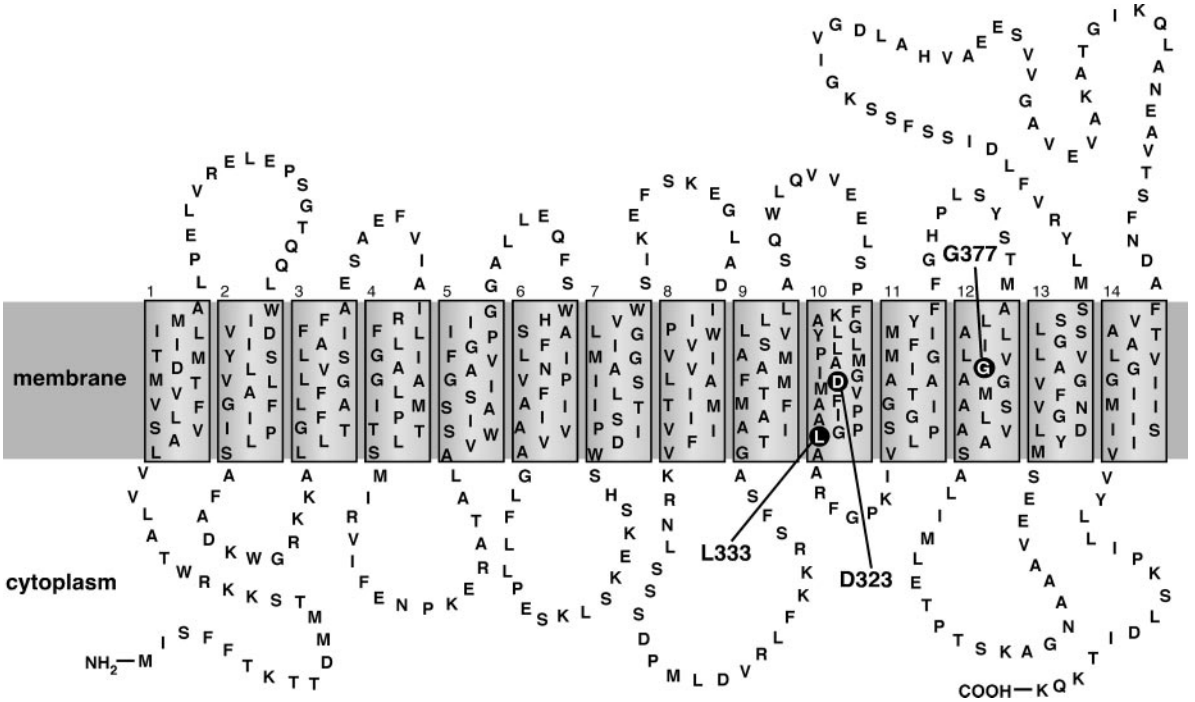

FIG. 1. Schematic topological representation of the QacA transport protein based on results of hydropathy analysis and limited solvent accessibility studies (11). The TMS are enclosed by gray shaded boxes and are numbered 1 to 14. The locations of D323 and L333 in TMS 10 and G377 in TMS 12 are indicated.

dequalinium conferred by these mutants was slightly above background (Table 1). In contrast, the G377E and particularly the G377D amino acid substitutions restored significant levels of dequalinium and chlorhexidine resistance and low levels of pentamidine resistance to strains with the D323C mutant protein (Table 1). Therefore, an acidic residue at amino acid position 377 in TMS 12 can functionally compensate for the absence of an acidic residue in TMS 10 to promote QacA-mediated bivalent drug resistance. Interestingly, the incorporation of G377C/D/E/Q substitutions in wild-type QacA caused a reduction in resistance to the majority of representative compounds (Table 1), suggesting that the amino acid residue at position 377 could be a general influence in determining the QacA multidrug recognition profile.

To further characterize the mutant QacA transport proteins, each was examined for its capacity to promote the efflux of representative monovalent (ethidium) and bivalent (DAPI [4′,6′-diamidino-2-phenylindole]) substrates from *E. coli*, as

TABLE 1. Relative expression and resistance capacities of QacA mutant proteins

QacA mutation	Relative expression (% of wild-type QacA)[c]	Relative MIC of substrate[a,b]					
		Monovalent			Bivalent		
		Et (dye)	R6G (dye)	Bc (Qac)	Dq (Qac)	Pe (Dd)	Ch (Bg)
Wild-type QacA	100	100 (250)[d]	100 (800)	100 (30)	100 (200)	100 (250)	100 (4.25)
No QacA	NA[e]	15	45	50	15	35	10
D323C	137	200	100	100	15	40	10
D323C-G377C	83	140	90	100	40	40	10
D323C-G377D	110	200	70	100	105	65	85
D323C-G377E	109	100	55	100	70	50	60
D323C-G377Q	123	180	65	90	30	40	15
G377C	74	40	70	100	75	50	50
G377D	110	70	70	100	70	40	50
G377E	108	60	55	75	40	35	25
G377Q	111	70	55	85	45	40	35

[a] MICs were determined in *E. coli* DH5α cells expressing QacA proteins as previously described (7). Values are percentages of the MIC for strains expressing wild-type QacA and are averages of at least two separate experiments.
[b] Abbreviations, Qac, quaternary ammonium compound; Dd, diamidine; Bg, biguanidine; Et, ethidium; R6G, rhodamine 6G; Bc; benzalkonium; Dq, dequalinium; Pe, pentamidine; Ch, chlorhexidine.
[c] Percentage wild-type QacA expression levels are the average of five Western blot analyses.
[d] Values in parentheses are resistance levels conferred by wild-type QacA (μg/ml).
[e] NA, not applicable.

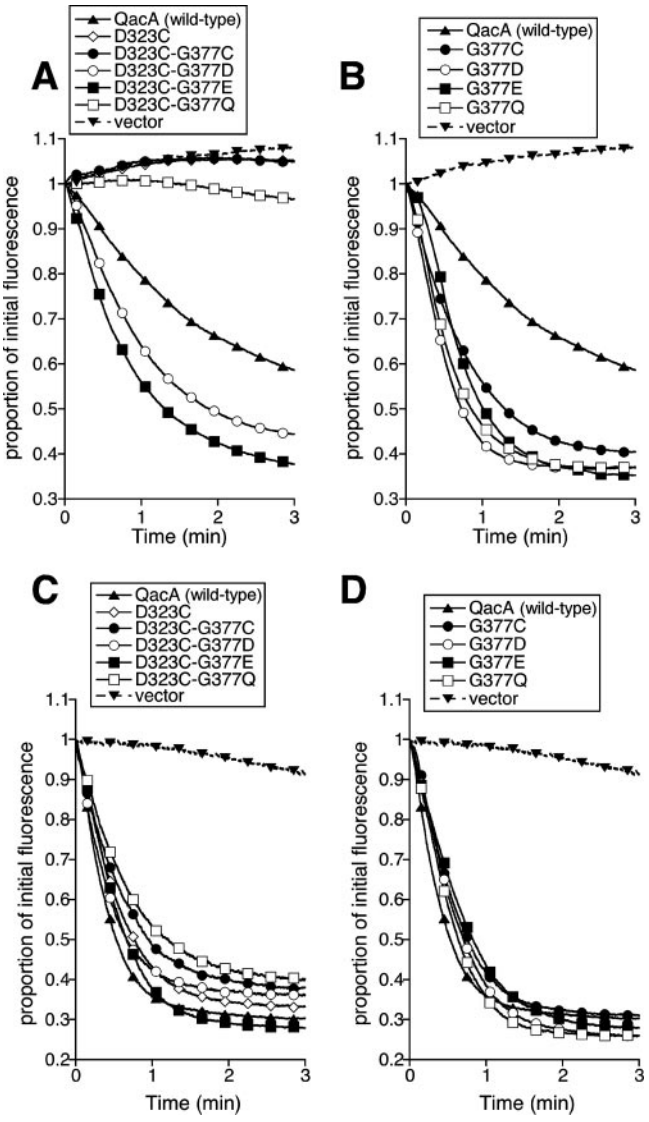

FIG. 2. Transport mediated by mutant QacA proteins. *E. coli* DH5α cells expressing G377 mutants of QacA D323C (A and C) or wild-type QacA (B and D) were loaded with either 8 μM DAPI (A and B) or 15 μM ethidium (C and D), and efflux was monitored fluorometrically. The curve depicting DAPI efflux mediated by the D323C mutant is partially obscured by the D323C-G377C QacA mutant curve (A). Each assay was conducted in at least duplicate, and results shown are from a representative experiment.

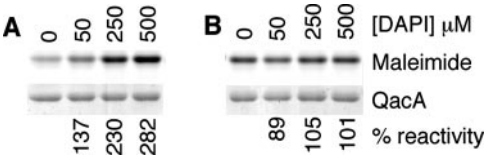

FIG. 3. Fluorescein maleimide reactivity studies of QacA mutant proteins in the presence of the bivalent substrate DAPI. The L333C (A) and D323A-L333C (B) QacA mutant proteins were reacted with fluorescein maleimide in the presence of 0, 50, 250, and 500 μM DAPI, purified by affinity chromatography, and fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as previously described (11). The fluorescence associated with each band is shown in the top panels, and the Coomassie blue-stained QacA proteins are shown in the lower panels. The percent reactivity to fluorescein maleimide of the DAPI-treated samples relative to the untreated sample for each mutant is indicated below each band.

previously described (7, 11). In line with its inability to confer resistance to bivalent cationic substrates, the D323C QacA mutant failed to mediate the transport of DAPI above background levels (Fig. 2A). However, the D323C-G377D and D323C-G377E QacA double mutants facilitated DAPI transport at an even higher rate than the wild-type protein (Fig. 2A). In contrast, the D323C-G377C and D323C-G377Q QacA mutants did not demonstrate significant DAPI transport activity (Fig. 2A). This implies that an acidic residue is required at position 377 to restore the capacity for bivalent substrate recognition to the D323C QacA mutant protein. In contrast to the reduced overall drug resistance capacity of the G377C/D/E/Q QacA mutants (Table 1), these proteins facilitated DAPI transport at a rate exceeding that of the wild-type protein (Fig. 2B), suggesting that these mutants may better accommodate DAPI in the binding site. Significantly, none of the G377 amino acid substitutions made in either the wild-type or D323C QacA backgrounds affected QacA ethidium transport rates (Fig. 2C and D).

Previous solvent accessibility studies of the QacA transport protein determined that amino acid positions at the C-terminal end of TMS 10 (e.g., position 333) could be made more accessible to solvent by the addition of DAPI, a finding which may reflect conformational changes around TMS 10 that may occur upon DAPI binding (11). To determine if an acidic residue in TMS 10, which is pivotal for DAPI binding, is integral to the DAPI-induced conformational switch involving TMS 10, a D323A-L333C QacA mutant was constructed and compared to the L333C QacA mutant for its reactivity with fluorescein maleimide in the presence and absence of various concentrations of DAPI, as previously described (11). The addition of 500 μM DAPI induced an almost threefold increase in fluorescein maleimide reactivity of the L333C mutant (Fig. 3A). However, DAPI had no noteworthy effect on the level of reactivity of the D323A-L333C mutant protein to fluorescein maleimide (Fig. 3B). This implies that DAPI does not bind to the D323A-L333C QacA mutant to induce a conformational change and an associated shift in the solvent exposure of position 333. Indeed, the D323A-L333C QacA double mutant also lacks the capacity for DAPI transport, a function maintained by the L333C mutant protein (data not shown). Similar studies were conducted to determine if mutations incorporated at position 377 would restore the capacity for a DAPI-induced conformational shift to the D323A-L333C QacA mutant. Although the DAPI transport capacities of D323A-L333C-G377C/D/E/Q triple mutants closely reflected those of the D323C-G377C/D/E/Q double mutants (Fig. 2A and data not shown), the fluorescein maleimide reactivity of these QacA triple mutants was unaffected by the addition of DAPI (data not shown). Therefore, even though the D323A-L333C-G377D/E QacA triple mutants were able to recognize and transport DAPI, the dynamics of DAPI binding may be distinct from those occurring within the L333C mutant, where DAPI may bind in an alternative orientation.

Given that D323 of QacA is likely to partake in electrostatic interactions with bivalent cationic substrates, the compensa-

tory effect of incorporating an acidic residue at position 377 of QacA after the neutralization of D323, implies that position 377 is placed within the QacA bivalent substrate binding region, where it can mediate similar interactions with substrates. This is the first time that a residue outside TMS 10 of QacA has been demonstrated to line the bivalent drug binding region and reflects the remarkable flexibility in drug binding interactions by QacA. Since position 377 is located away from TMS 10, it is likely to lie in a different spatial position within the bivalent drug binding region relative to D323. Therefore, bivalent cationic drugs may be bound in different orientations when stabilized by electrostatic interactions with an acidic residue at position 377 or 323. Electrostatic interactions in the multidrug binding site of the *E. coli* MdfA multidrug exporter have been shown to be similarly flexible (1). The incorporation of an acidic residue at position 335, which is likely to reside on a different face of the multidrug binding site than the important E26 (2, 5), can restore the capacity of a E26T MdfA mutant to recognize certain cationic substrates (1).

Interestingly, despite restoring a significant overall degree of bivalent cation resistance to the D323C QacA mutant, the level of drug recognition restored by the G377D/E mutations varied for different substrates and was particularly low in the case of pentamidine. Likewise, the ability of the MdfA E26T derivative to recognize different cationic substrates after the incorporation of an acidic residue at position 335 varied; for some cations no recognition was restored (1). This could be a reflection of physical constraints within the drug binding regions of these multidrug transporters. Although there is flexibility in the spatial organization of electrostatic interactions with some substrates, other parts of the binding site may be more rigid and restrict the orientation of subsets of substrates, such as pentamidine in QacA, preventing them from readily assuming stabilizing electrostatic interactions with acidic residues in novel spatial positions. If this is the case, it follows that with a reorganization of the position of important binding determinants, a multidrug transporter could rapidly adapt to bind new substrates which were previously unrecognized due to physical constraints in mediating stabilizing binding interactions. The observation that acidic residues exist in different positions in the bivalent drug binding site in naturally occurring QacA/B variants could be a manifestation of this phenomenon and reflect specific selective forces of different environments.

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